An update on potential biomarkers for diagnosing diabetic foot ulcer at early stage

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ABSTRACT

As one of major chronic complications of diabetes, diabetic foot ulcer (DFU) is the main cause of disability and death. The clinical diagnosis and prognosis of DFU is inadequate. For clinicians, if the risk stratification of DFU can be obtained earlier in diabetic patients, the hospitalization, disability and mortality rate will be reduced.

In addition to the inflammatory biomarkers that have been widely concerned and used, e.g., procalcitonin, pentraxin-3, C-reactive protein (CRP), interleukins (ILs), and tumor necrosis factor-α (TNF-α), etc., a more comprehensive prediction of the risk and severity of DFU is needed to reflect new biomarkers for therapeutic intervention effects. Along with the development of systems biology technology, genomics, proteomics, metabolomics and microbiome have been used in the studies on DFU for better understanding of the disease. In this review, new biomarkers that are expected to assist in the accurate diagnosis and risk stratification of DFU will be discussed and summarized in detail.

1. Introduction

Diabetes mellitus (DM) is a metabolic disease characterized by chronic hyperglycemia, caused by defects in insulin secretion and/or utilization [1]. It is reported by WHO that about 422 million people suffer from diabetes and it is expected that about 271 million more people will have diabetes by 2045 [2,3]. Until now, diabetes treatment drugs can only control blood glucose levels but cannot completely cure the disease. Diabetes mellitus may lead to chronic complications as the results of improper control. Various complications even happened at the first diagnosis of DM. 15–25 % of diabetic patients will suffer from diabetic foot ulcer (DFU), while DFU and amputation are the main causes of morbidity and mortality. 85 % of diabetic amputees suffer from DFU first, and then become serious gangrene or infection. Zhang et al. [4] found a pooled worldwide prevalence of diabetic foot ulceration of 6.3 % (95 % CI: 5.4–7.3 %), being more common in male diabetic patients than in female patients. The prevalence of new chronic diabetic foot ulcers increased from 20.7 to 33.1 per 1000 people ($P < 0.0001$) from 2003 to 2017. However, its prevalence varies in different countries and regions [5,6].

Since DM is associated with long-term complications of microvasculars and macrovasculars, it is necessary to detect the complications early. If the risk stratification of DFU can be obtained earlier in diabetic patients, the hospitalization, disability and mortality rate will be reduced. Defined as "characteristics of objective measurement and evaluation as indicators of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention", biomarkers are of great significance in early clinical diagnosis, disease prevention and disease progression prediction. It also plays an important role in discovering and developing new therapeutic drugs or evaluating the therapeutic strategies. Recent advances in molecular biology, genomics, transcriptomics, proteomics, metabolomics, and microbiomics have led to the identification of more biomarkers that can be used as potential candidates for clinical applications.

Abbreviations: AUCROC, area under the receiver operating characteristic curve; BAFF, B-cell activating factor; BCAAs, branched-chain amino acids; circRNA, circular RNA; CRP, C-reactive protein; DM, diabetes mellitus; DF, diabetic foot; DFI, diabetic foot infection; DFU, diabetic foot ulcer; DKD, diabetic kidney diseases; eNOS, endothelial nitric oxide synthase; EPCs, endothelial progenitor cells; ESR, erythrocyte sedimentation rate; ECM, extracellular matrix; RH, fast-healing; HC, healthy subjects as control; HSP, heat-shock protein; HBOT, hyperbaric oxygen therapy; HIF, hypoxia-inducing factor; IDFU, infectious diabetic foot ulcer; IFN, interferon; ILs, interleukins; LC–MS/MS, liquid chromatography-tandem mass spectrometry; LO, lysyl oxidase; LOX, lysyl oxidase family; MDC, macrophage derived chemokine; MMPs, matrix metalloproteinases; miRNAs, MicroRNAs; MVD, micro-vessel density; NETs, neutrophil extracellular traps; NH, non-healing; NIDFU, noninfected diabetic foot ulcer; PTCH1, Patched-1; WDFU, patients without diabetic foot ulcer; PTX-3, pentraxin-3; PCT, procalcitonin; PAD4, protein arginine deiminase 4; RUNX1, runtrelated transcription factor 1; SerpinB3, serine protease inhibitors b3; SNP, single nucleotide polymorphism; ST2, suppression of tumorigenicity 2 receptor; TARC, Th2-type cc chemokine and activation-regulated chemokine; TLR6, toll-like receptors 6; TNF-α, tumor necrosis factor-α; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; VEGF, vascular endothelial growth factor; WBC, white blood cell count.

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The progression of DFU can be predicted from targets and molecules related to the pathogenesis of DFU. In the research process of biomarkers, it is critical to correlate quantifiable biomolecules with clinical results, so that it has practical clinical application value. Based on clinical observations and biomarker evaluation, it can be used to guide clinical treatment methods. On the basis of understanding the physiological factors and the healing process of DFU (Fig. 1), some novel potential biomarkers have been revealed and even applied in clinical

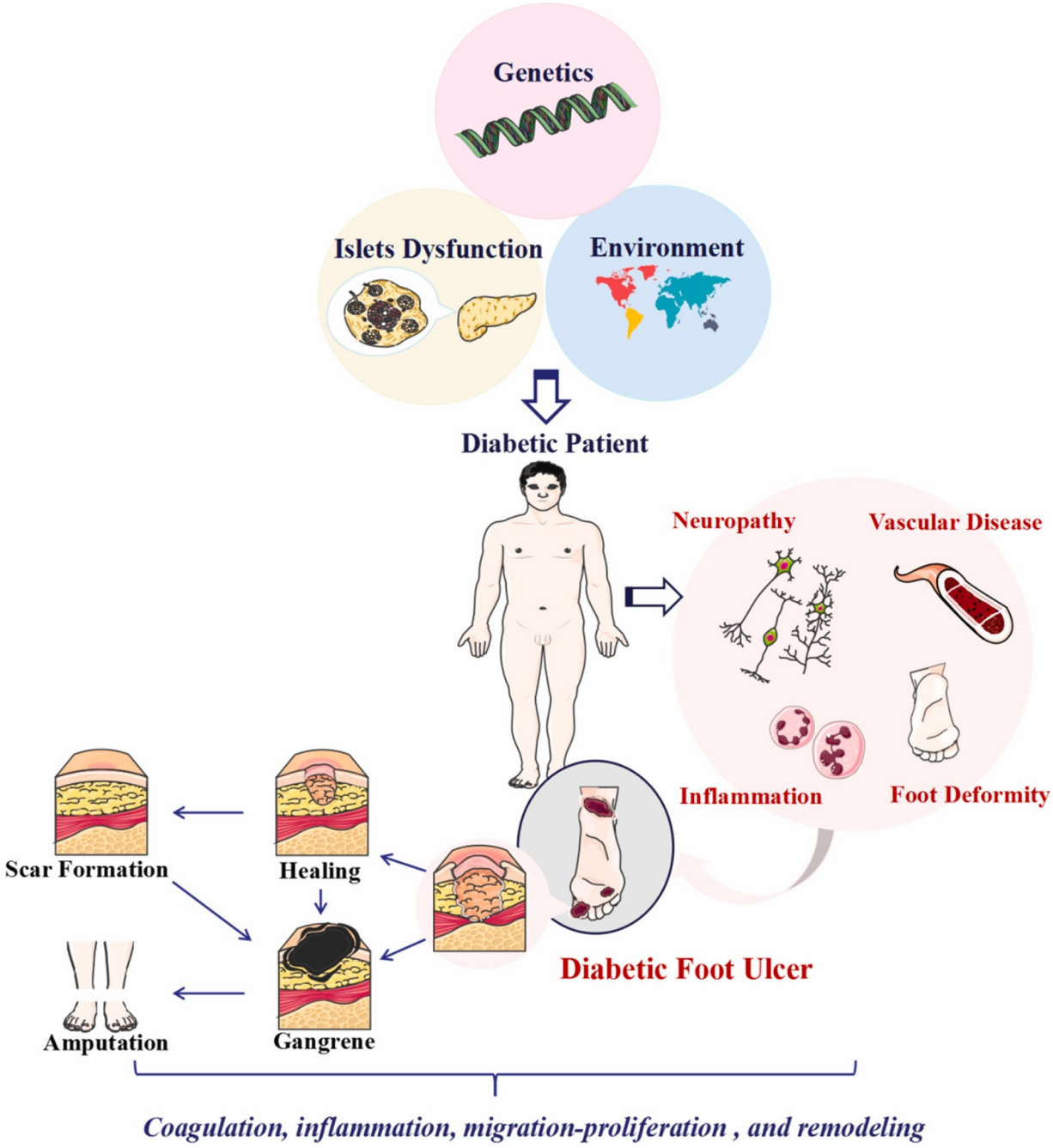

Fig. 1. Pathophysiological factors of diabetic foot ulcer (DFU) and its healing process. The occurrence of diabetes is related to the environment, genes, islet dysfunction etc. neuropathy, angiopathy, inflammation, foot deformity and other factors lead to DFU. In the process of ulcer healing, DFU might develop to scabs or gangrene. After the scabs formed, it may further deteriorate into gangrene, and later, gangrene may lead to amputation. The process of DFU healing can usually be divided into at least four overlapping phases: coagulation, inflammation, migration-proliferation (including matrix deposition), and remodeling.

practice during the research processes of DFU using molecular biology, genomics, proteomics, metabolomics and microbiomics (Table 1). In this article, we will focus on the advances in novel potential biomarkers of DFU using the above research platforms.

2. Pathological changes of DFU and its management

2.1. Pathological factors

The pathological changes of DFU are related to many diseases, among others, to hyperglycemia, peripheral neuropathy, peripheral vascular disease, foot deformity, sepsis, inflammatory cytokines, and infection susceptibility [7,8]. It's worth noting that most of these risk factors do not cause foot ulcers independently [9]. When the feedback loop between insulin action and insulin secretion fails to function properly, the role of insulin is affected in insulin-sensitive tissues (such as liver, muscle, and adipose tissue), so as to islet β-cell dysfunction. In T2DM, under a set insulin level, insulin resistance increases glucose production in the liver and reduces glucose absorption in muscle and adipose tissue. In addition, β-cell dysfunction leads to reduced insulin release. These changes will cause an increase in the level of blood glucose [3]. Among diabetic patients of poor glycemic control, hyperglycemia causes nerve fiber damages through a variety of mechanisms, e.g., chronic inflammation, advanced glycosylation end products formation, and protein kinase C formation [8]. As a result, peripheral neuropathy, including lesions in sensory, autonomic, and motor nerves [7], is the most common cause of DFU and accounts for 78 % of ulcer lesions [10]. Finally, neuropathy causes foot deformity or limited joint mobility, which lead to the enhancement of pressure in foot and consequently the formation of callus at the pressure spots. Callus tissue further increases the local pressure, which when combined with undetected repetitive lesions can cause local tissue injury, inflammation, tissue death (necrosis), and eventually ulceration [7]. DM patients have a higher incidence of atherosclerosis, thickened capillary basement membrane, arteriosclerosis, and endothelial hyperplasia. These risk factors affect blood supply to the arteries, resulting in ischemia of surrounding tissues, which further leads to DFU [11].

2.2. Wound healing and DFU

Wound healing is a complicated process that is usually divided into at least four overlapping phases: coagulation, inflammation, migration-proliferation (including matrix deposition), and remodeling. In the case of acute wounds, there is a smooth and orderly progress in these phases, leading to timely healing. In contrast, chronic wounds are stagnant in the inflammatory phase and unable to heal [12]. In continuous inflammatory state, macrophages are the main producers of proinflammatory cytokines in wounds. Due to its plasticity, macrophages play a key role in the transition from the inflammation phrase to the proliferation phrase. Through impairing the recruitment of monocytes to the wound, reducing phagocytosis and preventing inflammatory macrophages from turning into an anti-inflammatory state, diabetes destroys the function of macrophages. In its late stage, diabetes also hinders the function of keratinocytes and fibroblasts, resulting in damages to the wound epithelial cells [7,13]. According to clinical evidence and experimental research reports, unlike the overlapping linear progression of acute injury, DFU and other types of chronic wounds are in different phases at the same time in a certain area, and cannot follow the orderly and reliable progress of wound healing [14]. More than 100 known physiological factors delayed wound healing in DM patients, including the decreased or impaired growth factor, angiogenic response, macrophage function, collagen accumulation, leukocyte recruitment, extracellular matrix deposition, the amount of granulation tissue, the migration and proliferation of fibroblasts and keratinocytes, the quantity of epidermal nerves, bone healing, and the balance between the accumulation of extracellular matrix (ECM) components and their remodeling by matrix metalloproteinases (MMPs) [15,16].

2.3. Management of DFU

The etiological and pathological mechanisms of DFU are complicated, so its treatment method is often not only one, but it needs to be treated in multiple ways. The current standard of DFU care consists of four basic principles, namely infection management, debridement, pressure relief, and revascularization when necessary. Although these management principles are regarded as the gold standard for DFU care, the healing rate of DFU is still not ideal, so it is necessary to explore new therapies [17,18]. At present, in addition to these principles, diabetic foot care with multidisciplinary methods has already become the main treatment, including nonsurgical debridement agents, dressings and topical agents, hyperbaric oxygen therapy, negative pressure wound therapy, bioengineered skin substitutes, growth factors, stem cells, energy-based therapies (e.g. electrical stimulation, laser therapy, electromagnetic therapy, shockwave therapy, and phototherapy), and systemic therapies (e.g. vildagliptin, iloprost infusion, low-molecular-weight heparin, oral pentoxifylline, and many herbs) [19]. Borys et al. [20] enrolled 75 DFU patients, of which 53 received negative pressure therapy and 22 received primary treatment, and the result showed that negative pressure therapy on wound is safe for T2DM neurological, non-ischemic, and non-infectious DFU patients, but this observational study has not proven its effectiveness better than standard therapies. Multiple ways have been applied in the clinic, but some of these novel therapies currently still lack sufficient evidence to identify their clinical efficacy [19].

3. DFU biomarkers

3.1. Inflammatory biomarkers

3.1.1. Procalcitonin

Procalcitonin (PCT), a polypeptide released by thyroid C cells, liver, lung, and kidney parenchymal

cells, is composed of 116 amino acids as a precursor of calcitonin, which plays an important role in the diagnosis of bacterial infection as a biomarker [21].

Umapathy et al. [22] carried out a clinic study with 185 T2DM patients, and divided their into three groups, which were T2DM patients without DFU (WDFU), T2DM patients with infectious DFU (IDFU), and T2DM patients with noninfected DFU (NIDFU). The blood analysis was carried out subsequently and it was reported that PCT might be a key indicator for the diagnosis of IDFU patients. It showed that in IDFU patients, PCT (0.99) had the largest value of the area under the receiver operating characteristic curve (AUC_{ROC}), followed by C-reactive protein levels (CRP, 0.78), white blood cell count (WBC, 0.76), and erythrocyte sedimentation rate (ESR, 0.74). When the critical value was 0.5 ng/mL, the sensitivity of PCT was 54 %, and its specificity was 100 %, as well as for the diagnosis of IDFU, the positive predictive value was 100 %, and the negative predictive value of was 12 %. Besides, PCT levels in the peripheral circulation were positively correlated with T-helper-1- cell cytokines (e.g., interferon-γ and interleukin-28A) and subtype cytokines 17 (e.g., interferon-γ1 and interleukin-29). Moreover, comparing IDFU and NIDFU, the PCT levels difference between them was up to 7 times, better than the traditional indicators, i.e., CRP, WBC, and ESR, etc., and had higher sensitivity and specificity. Therefore, PCT can be used as an important biomarker for IDFU diagnosis. Massara et al. [23] enrolled 15 DM patients with IDFU and 15 DM patients with NIDFU, and the results showed that the PCT ($P < 0.00001$) and CRP ($P = 0.0009$) levels in the IDFU group were significantly higher than those in the NIDFU group, while ESR and WBC levels were also higher but with a minor statistical significance ($P = 0.4661$ and 0.0095, independently). Consequently, PCT levels had a higher efficiency in distinguishing IDFU from NIDFU. Park et al. [24] reported that PCT and CRP were significantly related to the degree of DFU infection. Interestingly, only PCT levels could

Table 1

Potential Biomarkers for Diagnosing Diabetic Foot Ulcer

Potential Biomarkers		Changes in DFUs (Compared with DMs)	Conventional Role	Assessments of Their Potentials in Diagnosing DFU	Reference
Inflammatory Biomarkers	PCT	↑	Playing a critical role in the diagnosis of bacterial infection as a biomarker.	An important biomarker for IDFU diagnosis in distinction of mild and severe IDFU, and differentiation of soft tissue infections and teomyelitis.	[21,22,23,24,25,26,27]
	PTX-3	↑	Inhibiting angiogenesis and restenosis; reflecting vascular inflammation directly.	A biomarker for determination of the disease progression of IDFU assisting in the decision of amputation; A candidate biomarker for vascular inflammation.	[28,29,30,31]
	CRP	↑	Acting as inflammatory biomarker especially in the acute phase and associated with DM.	An important biomarker for the distinction of Grade 1 and Grade 2 ulcers and the identification of infection or not. An independent risk factor for amputation.A prognostic factor for DFU patients of complete wound healing or mortality.	[26,32,33,34,35,36,37]
	IL-22	↑	Promoting wound repair in DM by improving keratinocyte pro-healing functions.	Can provide therapeutic benefit for DFU patients by accelerating the healing process of DFU.	[15,40]
	IL-20	↑			
	IL-24	↑			
	IL-18	↑/↓	One manifestation of inflammatory cascade and immune dysfunction.	An important biomarker for the diagnosis of DFU.	[34,41]
	IL-6	↑	Regulating immunity.	A candidate biomarker for determination of the disease progression of DFU.	[34]
	TNF-α (BAFF)	↑	Regulating immune cells and inducing insulin resistance.	A candidate biomarker for the differentation of IDFU and NIDFU.	[41]
Genomic Biomarkers	MiR-217	↑	Inhibiting the expression of target genes by modifying post-transcriptional mechanisms.	Promoting wound healing. Candidate biomarkers of wound healing.	[44,45,46]
	MiR-23c	↑			
	MiR-155	↑			
	MiR-126	↑			
	HIF-1 gene	↓	Regulating the expression of genes involved in angiogenesis, metabolic changes mediates adaptive cell response to hypoxia, proliferation, migration, and cell survival.	Candidate biomarkers of wound healing.	[50,51]
	LOX gene	↑	Playing an important role in dermal wound repair by regulating cell proliferation, metastasis and angiogenesis.		[52]
Proteomic Biomarkers	Elastase	↑	Promoting thrombosis, inflammation and endothelial function.	Candidate biomarkers for delayed healing.	[66]
	Histone	↑			
	Neutrophil gelatinase-associated lipocalin	↑			
	Protease 3	↑			
	SerpinB3	↓	Significantly up-regulated in DFU.	Candidate biomarkers for promoting healing.	[68]
	MDC	↑	Inhibiting the function of tumor immune system and promote the occurrence of tumor.		
	TARC	↑	A specific functional ligand for CCR-4 which is a receptor selectively expressed in T cells.		
	Clusterin	↑	Independent predictor of the progression of type 2 diabetic nephropathy.	Candidate prognostic biomarkers of DFU healing.	[72]
	Syndecan 4	↑	Affecting cell adhesion, proliferation, migration, and acting as the central medium of mechanical transduction.		
	S100	↑	Stimulating expression of inflammatory response.		
	MMP9	↑	Playing an important role in collagen degradation and tissue destruction.	Candidate biomarkers for delayed healing.	[69]
	Collagens CO15A1, CO18A1	↑	Anti-angiogenesis.		
	Tendon proteins	↑			
	MMP-8	↓	Non-competitive inhibitor.		[73]
Metabolic Biomarkers	Arginine	↓	Improving wound healing.	Candidate biomarkers of wound healing.	[70,71,73,77]
	Isoleucine	↓	Promoting muscle protein synthesis.		
	Leucine	↓	Involved in the formation of collagen and elastin.		
	Threonine	↓	Essential matrix for wound healing.		
Microbial Biomarkers	Gram-positive cocci (*Staphylococcus* and *Streptococcus* spp. etc.)	↑	Important in prognosis of infection.	The most common bacteria in DFI with short duration and superficial ulcers, and chronic DFI may have multiple bacterial infections Different aerobic and anaerobic bacteria can colonize DFU. *Bacterioides fragilis* is the largest number of anaerobic bacteria in DFIs.	[89,93,94,95]
	Anaerobic pathogens (*Bacterioides fragilis.* etc.)	↑			[89,90,91]

distinguish whether IDFU patients were co-infected. Velissaris et al. [21] concluded that PCT levels are conductive to diagnosing IDFU and NIDFU and distinguishing between soft tissue infections and osteomyelitis.

On the contrary, Korkmaz et al. [25] carried out a study in which they enrolled 38 IDFU patients, 38 NIDFU patients, and 43 WDFU patients, and the results showed that the levels of WBC, CRP, interleukins-6 (IL-6), ESR and fibrinogen of the IDFU patients were significantly higher than those of the NIDFU patients ($P < 0.01$). During the process of identification of IDFU and NIDFU, serum CRP has the highest AUC_{ROC} value (0.998; $P < 0.001$), while PCT had no significant effect. Based on the evidence of increasing of current data, serum IL-6 and fibrinogen are considered as two potential inflammatory biomarkers for the differentiation of IDFU. Some results also indicated that the predictive effect of PCT is less effective on patients with mildly DFU infection, but more effective on patients with severely IDFU [26,27].

3.1.2. Pentraxin-3

Pentraxin-3 (PTX-3) is a soluble pattern recognition receptor, which is synthesized by mononuclear phagocytes, fibroblasts, myeloid dendritic cells, granulosa cells, mesangial cells, endothelial cells, smooth muscle cells and adipocytes locally at the inflammatory site. Therefore, its level may more directly reflect vascular inflammation. In the process of inflammatory, a plenty of PTX-3 is produced in the vascular wall, which act as an endothelial regulator in thrombosis and ischemic vascular disease, and binds to angiogenic fibroblast growth factor-2, thereby inhibiting angiogenesis and restenosis [28,29].

Ozer et al. [30] concluded that PTX-3 is a valid marker for diagnosis of IDFU. The study selected 60 IDFU patients, 45 WDFU patients, and 45 healthy controls. According to the classification of clinical severity, IDFU patients were assigned to three subgroups: mild, moderate and severe. According the measurement of blood samples, the PTX-3 levels in the group of control, IDFU and WDFU were 5.83 (3.41–20), 1.47 (0.61–15.13) and 3.26 (0.67–20) ng/mL, respectively. Plasma PTX-3 were negatively correlated with blood glucose levels, and both of them were statistically different in all three IDFU subgroups ($P < 0.05$). In addition, there were only significant differences in the levels of CRP and ESR between patients with mild and moderate IDFU and between those of mild and severe IDFU ($P < 0.05$), while no significant difference between the moderate and severe IDFU. Takashi et al. [31] found that PTX-3 was positively correlated with presence of diabetes and glycosylated hemoglobin. Chronic hyperglycemia increased the serum level of PTX-3, which may indicated that PTX-3 was positively regulated by the blood glucose level. Nonetheless, the current evidence of PTX-3 as a biomarker of vascular inflammation is still insufficient, of which significance and function are still controversial and need further study.

3.1.3. C-reactive protein (CRP)

C-reactive protein (CRP), a hepatic-originated plasma protein, is a key element of any inflammatory reaction and a member of the pentraxin superfamily. As a result of tissue damage or infection, the plasma levels of CRP may rapidly increase to more than 1000-folds above normal values, therefore it is widely used a monitor marker in the acute phase, responding to tissue damage, infection and inflammation in routine clinic. Recently, it is a typical disease marker of cardiovascular diseases for its remarkable significance in clinic and pathology. The role of CRP, as a research and therapeutic reagent, has been fully clarified by the current booming of researches in various diseases and related conditions. Studies have shown that elevated CRP levels are associated with diabetes [32,33].

Weigelt et al. [34] found that acute DFU and its severity were associated with a significant increase of proteins, cytokines, and chemokines, but not concerned with the concomitant infection. Blood analysis was performed on 282 patients with acute DFU and 175 WDFU patients, and the result showed that patients with acute DFU had significantly elevation of CRP, fibrinogen, IL-6 compared with WDFU patients.

Metineren et al. [35] studied CRP levels of 56 amputees with DFU and gangrene before and after surgery. The retrospective study showed that 42 % of 56 patients (24) died within 2 weeks after surgery. The increase in mortality is accompanied by preoperative/postoperative CRP ≤ 1.5 ($P < 0.001$) and 73 years of age or older ($P < 0.001$), thus elevated serum CRP levels were key predictors of mortality. Two factors, CRP and old age, were reliable prognostic factors for DFU patients. A prospective study of Pickwell et al. [36] screened out 575 patients with IDFU, of whom 159 cases underwent an amputation ultimately. Statistical analysis of this study suggested that the elevated CRP levels can be identified as an independent risk factor for the amputation surgery in DFU patients.

Jeandrot et al. [26] enrolled 93 DFU patients without antibiotics treatment within six months before the study, which were divided into IDFU and NIDFU groups, and the control group consisted of 102 DM patients without DFU. The analysis of blood samples showed that CRP, as a single biomarker, was the most effective indicator for distinguishing Grade 1 and Grade 2 ulcers. From the perspective of immunology, the increase of CRP and PCT could be biomarkers of immune responses mediated by TNF -α, IL-6 and IL-1. In contrast, leukocyte and neutrophil counts had no predictive effect. Moreover, the most relevant combination obtained from logistic regression was the correlation between CRP and procalcitonin The ulcer grade could be more effectively identified by comparing receiver operating characteristic (ROC), which indicated that only the two biomarkers CRP and PCT may play an important role in distinguishing IDFU and NIDFU, to ensure the rational use of antibiotics.

Tecilazich et al. [37] studied 29 healthy subjects, 39 diabetic patients without DFU risk, 25 diabetic patients with DFU risk, and 29 DFU patients. The prospective follow-up of DFU patients for 12 weeks demonstrated that serum low baseline CRP and IL-1α levels were relevant to the wound's complete healing and may be biomarkers of DFU healing prognosis.

3.1.4. Interleukin

Interleukins (ILs), produced by blood monocytes and tissue macrophages, are a group of cytokines that are critical important for the function of the immune system – both innate and adaptive. When the body is infected, ILs are secreted onto target cells and bind to the signals on the surface that activate the target cells, altering cell behavior. ILs levels would be elevated in patients with DFU along with the development of insulin resistance, abnormal healing and decreased with the healing of ulcers [38–40].

Sabuncu et al. [41] selected 20 patients with acute DFU, 21 DM patients without DFU, and 21 healthy volunteers. Blood sample analysis showed that IL-18 in DFU patients and DM patients without DFU was significantly elevated compared to that in healthy subjects ($P < 0.001$ and $P = 0.020$). However, these findings so far have not shown whether the increase in IL-18 was the cause or consequence of DFU. Consequently, further research is required to prove the role of IL-18 within

ulcers [42]. Weigelt et al. [34] reported that IL-6 in patients with acute DFU was higher than that in diabetic patients without diabetic foot (DF) ($P < 0.01$). Studies by Kolumam et al. [15] also exhibited that IL-20 subfamily cytokines were up-regulated during the normal healing of wounds, and ILs, namely IL-20, IL-22, and IL-24 could all promote the wound healing of diabetic mice. Among them, IL-22 could promote the healing of blood vessels through formation, epithelialization and granulation tissue formation, thereby significantly accelerating the healing process of diabetic foot wounds.

3.1.5. Tumor necrosis factor-α

Tumor necrosis factor alpha (TNF-α) is one of the major inflammation regulator and a cell signaling protein identified as a key role player in the cytokines network. TNF-α, as a versatile mediator and an important member of the TNF family that is mainly generated by monocytes, macrophages and T-cells, could affect many biological pathways including the cellular/molecular process of adhesion, migration, angiogenesis and apoptosis. The main function of TNF-α is regulating the immune system via the activation of T cells, B cells and macrophages, thereby inducing the expression of other cytokines and cell adhesion molecules. In addition, the role of TNF-α in wound healing is implied by its significant expression in vascular endothelial cells. TNF- α has been reported to have an indirect angiogenic effect, depending on the synthesis of secondary mediators, e.g., vascular endothelial growth factor (VEGF). However, it is difficult to understand the dual angiogenesis (pre/anti) role of TNF-α under different conditions. Overall, in addition to several malignant tumors, TNF-α dysregulation has also connected with a variety of human inflammatory diseases [43]. It also induced insulin resistance by attenuating insulin signaling by promoting serine phosphorylation of insulin receptor substrate 1. B-cell activating factor (BAFF), also included in the TNF-α family [42–44], indicates the presence of dysfunction in B cells.

Dhamodharan et al. [45] selected 44 IDFU patients and 40 NIDFU patients. Blood levels of BAFF and CRP were measured by multiplexed bead-based cytokine immunoassays during the course of the chronic wound healing process. It was showed the AUC_{ROC} for BAFF (0.89; [95 % CI: 0.73–1.0]) was higher than that of CRP (0.68; [95 % CI: 0.61− 0.76]). The optimal diagnostic cutoff level for BAFF was ≥2.35 pg/mL with a sensitivity of 62.0 % and specificity of 85.7 %. In addition, it showed that the BAFF levels were significantly positively correlated with the CRP levels, the levels of interferon (IFN) family cytokines (such as IFN-α2, IFN-γ, IL-28A/IFN-λ2), and the levels of IL-10 family cytokines (such as IL-19, IL-22, and IL-26), as well as negatively correlated with IL-6 receptor family (such as gp130/sIL-6Rβ).

3.2. Genomic biomarkers

3.2.1. MicroRNAs

MicroRNAs (miRNAs), 18 to 22-nucleotide-long non-coding RNAs, regulate approximately one-third of the human genome at the post- transcriptional level. miRNAs participate in nearly all biological process such as proliferation, differentiation, apoptosis and development, but also act as an important role in the pathogenesis of many diseases. Most miRNAs are expressed independently. Due to various transports from cells such as exosomes, circulating miRNAs are stable and can be found in blood, saliva, cerebrospinal fluid, urine, and other human body fluids. As a potential non-invasive biomarker, circulating miRNA can be used to treat tumors, dyslipidemia, diabetes and cardiovascular diseases [46–48]. Moreover, in addition to representing potential biomarkers, miRNA can also be used as a new therapeutic agent in the modern clinics [49].

Lin et al. [50] investigated the serum levels of hypoxia-inducing factor (HIF)-1α, VEGF and miR-217 in DFU patients, simple DM patients, and healthy controls, as well as DFU rat models treated with miR-217 inhibitors and/or HIF-1α siRNA. The results showed that compared with DM patients and healthy controls, serum miR-217 levels in DFU patients were elevated, while the levels of HIF-1α and VEGF were decreased (all $P < 0.05$). HIF-1α is a direct target gene of miR-217. miR-217 inhibitors treated DFU rats had the ulcer area decreased in foot, the rate of ulcer healing accelerated, the inflammatory factors (IL-1β, TNF-α and IL-6) significantly reduced, as well as HIF-1α and VEGF (all $P < 0.05$). Meanwhile, the micro-vessel density (MVD) in foot dorsum wound tissues and the protein expressions of HIF-1α, VEGF, VEGFR2, endothelial nitric oxide synthase (eNOS), MMP-2, and MMP-9 (all $P < 0.05$) were remarkably increased. It was concluded that inhibition of miR-217 could modify the HIF-1α/VEGF pathways, and further advancing angiogenesis and the inflammatory state of DFU rats, thus effectively promoting the ulcerated wound healing. Emerging evidence indicated that miRNAs play a vital role in modification of angiogenesis, collectively known as "angiomiRs", and the miR-23 family belongs to angiomiRs related to various kinds of angiogenic factors, (e.g., SDF-1α) in tissue biopsies isolated from DFU patients. Amin et al. [51] selected 10 cases of healthy controls, T2DM patients, T2DM patients with NIDFU and T2DM patients with IDFU respectively, and found that in the circulation and tissue biopsies of IDFU patients, the angiogenic factor SDF-1α was significantly reduced. Furthermore, the tissue-specific expressions of miR-23a and miR-23b were decreased, while miR-23c was elevated in T2DM patients with IDFU, and correlation analysis demonstrated that SDF-1α was significantly negatively correlated with miR-23c. Overall, miR-23c may become a candidate of angiogenesis inhibitor by targeting SDF-1α. Gao et al. [52] found that there was an overexpression of miR-155 in endothelial progenitor cells (EPCs) of DFU patients and in those of healthy controls induced by high-glucose. miR-155 overexpression could enhance high glucose levels induced increases of lactated hydrogenase, apoptosis, types of reactive oxygen species, and reductions of cell viability, migration, tube formation, and nitric oxide production. The above biological processes could be restrained by the inhibition of miR-155. The 3' untranslated region of patched-1 (PTCH1), a receptor of the sonic hedgehog signaling pathway, was target by miR-155 directly, which also downregulated its mRNA and protein expression. These findings suggested that miR-155 may be a potential therapeutic target for DFU. Zhang et al. [53] found that miR-126 mRNA expression in peripheral blood of DM patients was remarkably decreased compared to the healthy controls ($P < 0.001$). In addition, its expression in DFU patients was further reduced ($P < 0.001$), indicating that miR-126 mRNA may be an attractive biomarker and therapeutic target for future treatment of DFU. Zhao et al. [54] analyzed serum circular RNA (circRNA) and mRNA expression profiles for DF patients (n = 3) and DM patients (n = 6). The results showed that DF patients had 279 mRNAs upregulated, 353 mRNAs downregulated, and 33 circRNAs differently expressed compared with DM patients. Toll-like receptors 6 (TLR6), runt-related transcription factor 1 (RUNX1) and the suppression of tumorigenicity 2 receptor (ST2) were found to be related to the progression of DM and DF, and lysosomal pathway plays a key role in the occurrence and development of DF. In addition, this study was the first to observe methane metabolism and Chagas disease pathways in the occurrence and development

of DF. These findings provided clues to the molecular mechanism of DF at the level of circRNA and mRNA.

3.2.2. Gene polymorphisms

The discovery of gene polymorphisms could give an indication of the incidence and development trajectory of the disease, and help to understand the genetic susceptibility of the population. Genetic make-up can determine inflammatory and healing responses. As one of the major diabetic complications related to trauma, peripheral neuropathy and peripheral vascular disease, DFU patients have a wide range of gene variants in gene polymorphisms.

Mir et al. [55] found that heat-shock protein (HSP) 70 gene polymorphism, the HSPA1B genotype, was associated with the severity of DFU and the outcome of surgical treatment. Zubair et al. [56] investigated the potential role of C2437T (Met493Thr) single nucleotide polymorphism (SNP) of HSP 70 among DFU patients, DM patients, and health control (N = 50 in each group) in a prospective cohort study. The results demonstrated that in DFU patients, homozygous and heterozygous hspHSP70 hom gene polymorphisms were significantly positively correlated with the T allele. Soroush et al. [57] found there is a significant association between the Vok gene FokI functional variation and DFUs in the Iranian population. Increased levels of TBARS in patients carrying the T allele of the FokI polymorphism indicated that this mutation is associated with oxidative stress in diabetic patients. In addition, Amoli et al. [58] found that functional polymorphisms in the VEGF gene were associated with DFU. The lower A allele frequency in DFU patients has a protective effect, which might be due to increased angiogenesis in patients carrying this allele. Li et al. [59] also confirmed this result in the Chinese Han population in a case-control study of 88 T2DM patients versus 97 DFU patients, and found individuals carrying VEGF rs699947 A allele that were less susceptible to DFU in the Chinese Han population.

In recent years, emerging pieces of evidence showed that the increase of hypoxia conditions and hypoxia caused abnormal cellular responses are important pathogenic factors which cause the wound healing of DFU delayed [60]. HIF-1 (a heterodimer of HIF-1α and HIF-1β) is the main regulator of oxygen homeostasis. HIF-1 intervenes adaptive cellular response to hypoxia by regulating the gene expression related to metabolic alteration, angiogenesis, proliferation, migration, and cell survival [61,62]. Hyperglycemia induces the unstable status of HIF-1α and suppresses its function, which will further inhibit HIF-1 signaling. Any means to elevate the expression or activity of HIF-1α are helpful to accelerate wound healing in experimental diabetes via the promotion of angiogenesis, EPC recruitment, and granulation [63,64]. Therefore, HIF-1 signaling is the potential target for diabetic wound treatment. Pathways related to HIF-1α regulation may have an important role in the underlying mechanisms of impaired diabetic wound healing, and the further understanding of these pathways will provide the base for developing novel therapeutic approaches in DFU treatment [61]. Pichu et al. [65] analyzed the HIF-1α gene polymorphism in DM patients with and without DFU (79 DFU patients, 79 T2DM patients, and 66 healthy controls), as well as p582 s (rs11549465) in Exon-12 of HIF-1α gene in order to illustrate its expression under these pathological conditions. Semi-quantitative RT-PCR results showed that the expression of HIF-1α gene in DFU patients was lower than those of the T2DM and control group, suggesting its possible role in the pathogenesis of DFU. In addition, compared with healthy controls, HIF-1α gene polymorphism existed in DFU patients.

Defined as cuproenzymes, lysyl oxidase family (LOX; also known as protein lysine 6-oxidase) is mainly composed of lysyl oxidase (LO) and lysyl oxidase-like 1–4 (LOXL1-LOXL4), and acts a pivotal part in stabilizing the extracellular matrix, especially the cross-linking of collagen and elastin, and further promotes wound healing by regulating cell proliferation, metastasis and angiogenesis. Pichu et al. [66] studied the relevance of LOX gene polymorphism G473A, G >A, (rs1800449) on the exon 1 of the LOX gene of T2DM patients with and without DFU and the impact of its expression on DFU. 906 samples were selected for genotype analysis, and there was a significant increase of 'A' allele in T2DM and DFU patients compared with the healthy subjects as control (HC), Allele wise analysis showed that the 'A' allele was present more frequently in T2DM (36.23 %, OR 1.069, P = 0.29) and DFU patients (41.69 %, OR 1.195, P = 0.003) than HC group (33.17 %). RT-PCR results showed that compared with the AA genotype of T2DM patients and the HC group, the transcription levels of LOX gene of DFU on the AA genotype were significantly increased. From these findings, it can be concluded that compared with HC, T2DM and DFU patients were associated with LOX gene polymorphism (G473A).

3.3. Proteomic biomarkers

In the past decades, with the impressive development of novel proteomics strategies, substantial progress has been made in the early-stage disease diagnosis and accurate prognosis. Although the standardization of proteomic approaches remains difficult, and the heterogenic property of proteins in various tissues leads to incomplete populations of the entire proteome, the advances of this technological field will expand our knowledge of disease biology in a large scale, discover new biomarkers or biomarker panels, open a new window for more effective early diagnosis, disease surveillance, and move towards personalized medicine.

Neutrophils, the most abundant circulating leukocytes in humans, can engulf and kill invading microbes and therefore represent the first defense line of the innate immune response. Extracellular fibres formed by granule proteins and chromatin released upon activation are called neutrophil extracellular traps (NETs). The production of NETs is a special death of neutrophils. This process is called NETosis. The main components of NETs include histones, DNA, and neutrophil elastase, which are caused by infectious factors and other inflammatory factors. High glucose levels were found to induce NETosis, and NETs were involved also in the pathogenesis of diabetes. NETs were described as drivers of diabetic complications, including diabetic wound and diabetic retinopathy [67]. NETosis is a natural defense process, but excessive or dysfunctional NETosis can also promote thrombosis, inflammation and endothelial function obstacle. Compared with normal people, the levels of circulating mononuclear and oligonucleosomes, cell-free double-stranded DNA, and neutrophil elastase elevated significantly in T2DM [68,69]. Using proteomics, Fadini et al. [70] found that NET components (elastase, neutrophil gelatinase-associated lipocalin, protease 3, and protein) were increased in nonhealing DFUs, and the neutrophil elastase level was elevated in wound along with the infection and delayed healing. Protein arginine deiminase 4 (PAD4) promotes neutrophils undergoing histone citrullination. In the skin of diabetic mice, PAD4 activity was increased and NETosis occurred in the bed of excisional wounds. Inhibition of PAD4 in diabetic mice reduced the formation of neutrophils and promoted wound healing. In conclusion, NETosis delays DFU healing. In addition, Fadini et al. [71] selected 75 DFU patients and set subgroup of non-healing (NH, n = 11) and fast-healing (RH, n = 17). Proteomic analysis showed there was differential expression of 92/286 proteins between NH and RH, which were related to the pathways of epithelial differentiation, protease inhibition, apoptosis, serine endopeptidase

activation, coagulation and regulation of defense response. In addition, serine protease inhibitors B3 (SerpinB3) in the fast-healing group was significantly up-regulated in the wounds of RH compared to that of NH, and further confirmed as biomarkers of promoting wound healing in a cohort study of 47 patients for independent validation. Wang et al. [72] carried out studies on the circulating proteins of DFU patients after healing to explore the prognostic biomarkers of DFU. The results showed that compared with the DFU group, macrophage-derived chemokine (MDC), Th2-type CC chemokine and activation-regulated chemokine (TARC), Clusterin and Syndecan 4, were elevated in DFU-treated group and similar to those in the health control group, which were prognostic biomarker candidates of DFU healing.

During the complex and highly regulated process of wound healing, proteases, e.g., MMPs are released for the degradation of gelatins and collagens, which can remove cells and necrotic tissue of damaged or dead from the injured site. Using multidimensional protein identification technology MS/MS, Krisp et al. [73] analyzed the protein composition of chronic diabetic foot exudates from 10 DFU patients, compared with those from split-skin donor sites of 6 patients with thigh degree II to III burns without any other disease. The study found that the expression of inflammatory response stimulating protein S100 increased and the amounts of MMP-1, MMP-2, and MMP-8 elevated. In addition, expression levels of proteins with anti-angiogenic properties, such as collagens CO15A1, CO18A1, and tendon proteins were increased in chronic DFU wounds. Jones et al. [12] observed the active MMP-8 and MMP-9 in diabetic wounds in mice and humans, and MMP-9 plays a harmful role in the healing of diabetic wounds, while MMP-8 is beneficial.

3.4. Metabolic biomarkers

Metabolomics, an approach of multidimensional profiling of small metabolites within biological samples, such as body fluids, cells, tissues, or organisms, has been experienced rapid technological development in the past two decades. These advances have promoted the application of metabolomics in the determination of predictive biomarkers for the incident and pathophysiologic mechanisms of diseases, especially those related to metabolic dysfunction [74,75]. Serum, plasma and urine are the most commonly used samples for metabolomics analyses, which can identify and quantify the endogenous metabolites of systemic metabolic processes Serum or plasma metabolomics assesses circulating metabolites, while urine metabolomics is used for the discovery of non-invasive biomarkers via metabolic end-products excreted by the kidney. Existing research evidence suggests that individuals with T1DM and T2DM generally alter metabolites such as glucose, fructose, amino acids, and lipids. Besides, these metabolites showed significant prospective and predictive associations with prediabetes, T1DM and T2DM, therefore metabolomics techniques can benefit to identify these internal metabolites and biomarkers [76]. Furthermore, metabolomics has been used to help understand DFU at the biochemical level and promote its understanding and management.

As both of the DFU and diabetic kidney diseases (DKD) are diseases have microvascular dysfunction features, Harrison et al. [77] carried out urinary metabolomics studies on DFU patients undergoing a 6 weeks of hyperbaric oxygen therapy (HBOT) to provide the underlying therapeutic rationale for application of HBOT in DKD. The results indicate that HBOT can reduce biomarkers of renal injury, oxidant stress, and mitochondrial dysfunction in patients receiving HBOT for DFU.

For evaluation and identification of specific amino acids related to wound healing outcomes of DFU patients, Hung et al. [78] utilized liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay on targeted metabolomic approach to analyze blood samples of 57 DFU patients, among of which 19 had unhealed and 38 had healed ulcers. The results showed that serum levels of arginine, isoleucine, leucine and threonine in the healed group were remarkably elevated compared with those of the unhealed group. Arginine is a basic amino acid and is an essential matrix in the process of wound healing. Numerous studies have shown that arginine can improve wound healing [79,80]. Isoleucine and leucine are branched-chain amino acids (BCAAs) that accelerate muscle protein synthesis [78]. The amino acids related to wound healing in DFUs are not same as those reported in traditional traumatic wounds. These findings verified that it's necessary to carry out large-scaled researches on the application of these amino acids in the treatment of DFU as indicators for prognosis or supplement regimens in the future.

3.5. Microbial biomarkers

A large number of bacteria, archaea, viruses, and single-cell eukaryotes live in the human body, and the microorganisms that coexist with the host are called microbiota. It regulates the early development of physiological host and has functions of regulating nutrition, immunity at all ages of the host [81,82]. In addition to the microbiota environment on the body surface, there is a complex metabolic interaction between the intestinal microbiota and the host. Numerous studies on the potential role of gut microbiota in the metabolic conditions, such as diabetes [83–86], have been carried out. Ralston et al. [87] highlighted a new connection between diet, inflammatory signaling and the gut microbiome. Animal studies support causal relationship between gut microbiome in the development of T2DM, insulin resistance and obesity [88, 89]. It has been suggested that short-chain fatty acids-producing bacteria may augment glucose stimulated insulin response and are typically reduced in T2DM [90].

For DFU, the microbiome studies are mainly focused on the wound site. A variety of bacterial communities, identified in DFU and diabetic foot infection (DFI), play an important role in the prognosis of infection. Depending on the combination of bacterial culture and molecular technologies, DFUs/DFIs can be colonized by various aerobic and anaerobic bacteria. DFIs of shorter disease procession seem to have a simpler microbiome that mainly colonized by Gram-positive *cocci* (*Staphylococcus* and *Streptococcus* spp.), while DFIs with a longer disease procession may have infections with a more complicated microbiome that can be colonized by various types of aerobic bacteria, including *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Pseudomonas* spp., etc. and anaerobic pathogens [91]. *Bacteroides fragilis* is the largest number of anaerobic bacteria in DFIs [92,93]. It has been reported that the abundance of anaerobic bacteria is limited and has little effect on the progress of infection [94]. In superficial ulcers, the microbiome is mostly composed of Gram-positive *cocci*, including *Staphylococcus aureus*, *Streptococcus pyogenes, β*-hemolytic streptococci or coagulase-negative staphylococci. Patients with deeper ulcers or non-naive antibiotics may be infected with enterococci, *Pseudomonas* or anaerobic bacteria [95,96,85].

In addition to the microorganism at the site of the wounds, the intestinal microorganisms are also related to DFU through their influence on the development of diabetes, including energy metabolism, inflammation, the innate immune system, and the bowel function of the intestinal barrier [97–99]. These results implied that changes in the gut microbiota may protect patients with DFU from antibiotic-induced

overgrowth of infection-associated bacteria, e.g., *Clostridium difficile* [95].

Microbiomics analysis and its association studies are revolutionizing clinical researches by providing more patient stratification and new biomarkers for the disease [100]. Further research on the gut microbiota and gastrointestinal tract in diabetic patients and DFU patients may further our understanding of their role in DFU.

4. Conclusions and future perspectives

Considering the increasing incidence of diabetes and its complications worldwide, it is necessary to conduct research on potential markers of diabetic foot with integration of multiple approaches. In recent years, great progress has been gained in understanding the pathogenesis and treatment of diabetic foot problems. DFU biomarkers, as the representative characteristic properties of the pathogenesis of diabetic wounds, can be helpful for further understanding of DFU, improving early clinical diagnosis, disease prevention, disease progression prediction and even treatment evaluation of DFU. Biochemical and molecular targets revealed by modern biotechnology approaches can be used as novel potential biomarkers for DFU, which may be applied in the diagnosis of DFU of the early stage after further researches from bench to bedsides, leading to better treatment and thus preventing the trauma of amputation.

Researches on disease biomarkers continue to develop rapidly in many fields. For instance, there are some physical biomarkers that are valuable and have already been considered for the diagnosis, risk stratification or follow up of DFU [101–103]. Ideally, combine novel markers with clinical observations and compare these results with the best routine tests as current clinical benchmarks, and even quantitative methods can be used to validate the correlation between these novel potential markers and the clinical observations of DFU, avoiding exaggeration of the value of the new platform. As far as DFU is based on complex systemic diseases, multiple factors related to potential clinical relevance, collaboration, validation, and consistency of clinical evidence should be considered. In short, the study of biomarkers in DFU is still in its early stage, and continuous attempts in this field will help reveal new insights into DFU treatment and improve its prevention and treatment levels.

Author contributions

The authors' responsibilities were as follows: Y.W. completed the collection and analysis of relevant literature and the preparation of the first draft of the thesis; T.S., J.W., X.D., and X.H. participated in the analysis and collation of the literature. Y.C. critically revised the manuscript for intellectual content; M.Z. and C.Z. are the project creators and principals, guiding the paper writing. All authors read and agree to the final text.

Declaration of Competing Interest

The authors report no declarations of interest.

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